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Zoological Research

Comparative Study of the Biological Activities of the Skin Secretions from Six Common Chinese Amphibians

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Abstract: Water-soluble skin secretions of six common Chinese amphibians were studied for their biological and enzymatic activities. The skin secretions of *Tylototriton verrucosus*, *Bombina maxima*, and *Bufo andrewsi* were found toxic to mice with the intraperitoneal LD_{50} of 11.5 mg/kg, 18.8 mg/kg, and 264 mg/kg, respectively. No acute lethal toxicities were observed for the skin secretions of *Rana nigromaculata*, *Rana guentheri* and *Rana limnocharis* in a dose up to 500 mg/kg. The lethal toxicities of the skin secretions of *T. verrucosus* and *B. maxima* to mice are in the same grade as those of Viperidae snake venoms. The toxic components in *T. verrucosus* and *B. maxima* skin secretions are the proteins with molecular weights ranging from 3 to 60 kDa. All the skin secretions had both proteolytic activity and trypsin inhibitory activity. The skin secretions from *T. verrucosus*, *B. maxima* and *B. andrewsi* also displayed wide spectrum antimicrobial activity. On the other hand, the skin secretions from *B. andrewsi* and *B. maxima* showed cytotoxicity on human cancer cells. All the six samples had not significant effects on mammalian blood coagulation system. Phospholipase A_2 activity was only found in the skin secretions of *T. verrucosus*. None of these skin secretions showed acetylcholine esterase activity.

Key words: Amphibian; Toxicity; Antimicrobial; Skin secretions; Biological activity

六种常见两栖类动物皮肤分泌物的生物活性比较

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摘要: 对6种常见两栖类动物皮肤水溶性分泌物的生物学活性与酶学活性进行了研究。其中, 红瘰疣螈(*Tylototriton verrucosus*)、大蹼铃蟾(*Bombina maxima*)、华西蟾蜍(*Bufo andrewsi*)皮肤分泌物对小白鼠具有致死毒性, 对小白鼠腹腔注射的半数致死剂量分别为11.5、18.8和264 mg/kg。而沼蛙(*Rana nigromaculata*)、泽蛙(*Rana guentheri*)、黑斑蛙(*Rana limnocharis*)的皮肤分泌物在小白鼠腹腔注射剂量达到500 mg/kg时, 仍不显示致死毒性。红瘰疣螈、大蹼铃蟾皮肤分泌物的毒性成分为3~60 kDa的多肽与蛋白质。6种两栖类动物皮肤分泌物都具有蛋白酶水解活性与胰蛋白酶抑制活性。红瘰疣螈、大蹼铃蟾和华西蟾蜍皮肤分泌物具有广谱抗菌活性。大蹼铃蟾、华西蟾蜍皮肤分泌物具有肿瘤细胞细胞毒活性。6种皮肤分泌物对哺乳类血液凝固系统无显著影响。仅发现红瘰疣螈皮肤分泌物具有磷脂酶A_2活性。6种皮肤分泌物均无乙酰胆碱酯酶活性。

关键词: 两栖类动物; 毒性; 抗微生物; 皮肤分泌物; 生物活性
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Amphibians are cold blooded vertebrates having a smooth or rough skin rich in glands. Amphibian skin is morphologically, biochemically and physiologically complex organ which fulfills a wide range of functions

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necessary for the amphibian survival, including respiration, water regulation, anti-predator, antimicrobial defense, excretion, temperature control, reproduction etc. (Clarke, 1997). The functional diversity of amphibian skin implies the biochemical diversity, and one of the major sources of biochemicals in amphibians is their skin gland secretions. The main components of the secretions include biogenic amines, steroids (bufotoxins), alkaloids, proteins and peptides (Daly *et al.*, 1987). The extraordinary complexity of biochemicals found in amphibian skin secretions, coupled with the high probability of their novel molecular structure compounds and clinically useful function, makes the amphibians an important target group (Clarke, 1997). Some amphibian skins have been widely used in traditional Chinese medicine. Dried and powdered toad skin secretions, known as "Chan Su", have the functions of detoxifcation, detumescence and acesodyne. Over the past several decades, numerous studies have focused on the bioactive components existed in amphibian skin secretions, especially peptides with diverse biological activities, including mammalian central and peripheral nervous system analogues, antimicrobial peptides (Bevins & Zasloff, 1990; Erspamer *et al.*, 1985; Lazarus & Attila, 1993). Interestingly, peptides which are highly homologue with snake venom components, like xenosin and Bv8 peptide, have also been characterized from amphibian skin secretions (Macleod *et al.*, 1998; Seon *et al.*, 2000; Mollay *et al.*, 1999).

Comparatively, a few reports in literature are documented for the study on the lethal toxicity of amphibian skin secretions, enzymatic activity, protease inhibitory activity etc. Here, we describe the systematic examination of the biological activities of water-soluble skin secretions from six common Chinese amphibian species, including their lethal toxicity on mice, enzymatic activity, antimicrobial activity, cytotoxicity on human cancer cells and effects on mammalian blood coagulation system. The animals collected and their taxononic positions are as the following: one Salamandridae salamander, *Tylototriton verrucosus*; one Discoglossidae toad, *Bombina maxima*; one Bufonidae toads, *Bufo andrewsi* and three Ranidae frogs, *Rana nigromaculata*, *Rana guentheri* and *Rana limnocharis*.

1 Materials and Methods

1.1 Materials

The chromogenic *p*-nitroanilide (*p*NA) substrates, H-D-Phe-Pip-Arg-*p*NA (S-2238), H-D-Pro-Phe-Arg-*p*NA (S-2302) were obtained from Kabi vitrum (Stockholm, Sweden); bovine trypsin, human thrombin, elastase, papain, thermolysin and casein were purchased from Sigma (St. Louis, USA). Dulbecco's modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were also from Sigma. All other reagents were of the highest purity available.

Kunming mice were from Kunming Institute of Zoology, the Chinese Academy of Sciences. During these experiments, mature amphibian animals were captured from southwest of China. They were *T. verrucosus* (from Chuxiong, Yunnan), *B. maxima* (from Chuxiong, Yunnan), *B. andrewsi* (from Neijiang, Sichuan), *R. nigromaculata* (from Neijiang, Sichuan), *R. guentheri* (from Neijiang, Sichuan) and *R. limnocharis* (from Neijiang, Sichuan).

1.2 Collection of amphibian skin secretions

The amphibian animals were first washed by water to remove contaminants from their skin surface, and then stimulated for 1-2 min by volatilized anhydrous ether immersed in absorbent cotton. During this process, animal skin surface was seen to exude copious secretions, and the animals were washed by distilled water to collect skin secretions. The collected samples were quickly centrifuged and the supernatants were frozen and lyophilized. The dried powder samples of amphibian skin secretions were stored at −20℃. The concentration of the secretion sample used in all the assays was determined by dissolving a certain amount of the dried powder in a certain volume of designed media.

1.3 Acute lethal toxicity on mice

Approximate intraperitoneal (i.p) LD_{50} was determined mainly according to the procedure of Meier & Theakston (1986). The tested sample was injected in-

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traperitoneally into mice weighing (20 ± 2) g (either sex). For each tested sample, mice were divided into three groups (eight to ten mice in each group) and were injected with different doses of the sample. The tested animals were observed for 24 h after injection. Survival times (time between injection and death) were recorded and the LD_{50} value was calculated. The thermal stability of the sample on the lethal toxicity was also tested by injection of the sample that had been incubated at 100℃ for 10 min.

1.4 Enzymatic activity assay

Proteolytic activity was assayed according to the method of Saleemaddin *et al*. (1980). One unit of the enzyme is the protein amount that causes a decrease of 0.01 absorbance unit in 10 min at 595 nm. Phospholipase A_2 activity was tested according to the method of Hass *et al*. (1968). The method of measuring acetylcholine esterase activity described by Berman (1973) was employed to measure acetylcholine esterase activity of the samples.

1.5 Protease inhibitory activity

Protease inhibitory activity of the sample was assayed by testing the inhibitory effects on trypsin and thrombin. Two chromogenic *p*-nitroanilide substrates, H-D-Phe-Pip-Arg-*p*NA (S－2238) and H-D-Pro-Phe-Arg-*p*NA (S－2302), were used for thrombin and trypsin, respectively. The assay was performed in 50 mmol/L Tris-HCl, buffer, pH 7.8. Different amounts of the sample tested were pre-incubated for 10 min at room temperature with trypsin or thrombin (final concentration, 0.5 μg/mL, 2 μg/mL, respectively). The reaction was initiated by the addition of the substrate (final concentration 0.5 mmol/L), and the formation of *p*-nitroaniline was monitored continuously at 405 nm for 2 min as described by Zhang *et al*. (1995).

1.6 Effects on blood coagulation

Citrated platelet-poor human plasma and platelet-rich human plasma (200 μL) were incubated at 37℃ for 1 min, then a 20 μL aliquot of diluted secretion samples was added and the clotting time was recorded. In some cases, 5 μL of $CaCl_2$ (final concentration 10 mmol/L) was added simultaneously with the secretion samples. Coagulating activity was determined by measuring the clotting time of purified human fibrinogen (0.5%) in 50 mmol/L Tris-HCl, pH 7.8, containing 0.1 mol/L NaCl. Fibrinogen (200 μL) was incubated for 2 min at 37℃ before addition of 20 μL of diluted samples.

1.7 Hemolysis and hemorrhagic activity assays

The hemolysis assay was carried out following Bignami's method (Bignami, 1993). Sterile phosphate buffer saline (pH 7.2) was supplemented with 0.1% (w/v) bovine serum albumin, 1 mmol/L calcium chloride, and 1 mmol/L sodium tetraborate. Whole blood from anesthetized male rabbit was collected by cardiac puncture and diluted in saline. The red blood cells were separated from plasma by centrifugation, wash once with saline, and suspended at a 1% cell concentration. 50 μL of blood red cell suspension and 50 μL of the sample were added to the wells of sterile round-bottom microwell plates. After incubation at 37℃ for 4 h, the plate was centrifuged at 1 500 *g* for 2 min. A 75 μL aliquot of each supernatant was transferred to a microwell plate and measured for absorption at 595 nm.

The method of Ownby *et al*. (1978) was used to assay hemorrhagic activity, subcutaneously injected a dose of 10 μg/g body weight of mouse. Six hours later, the mice were sacrificed to measure the area of hemorrhagic spots.

1.8 Antimicrobial assays

The standard bacteria and fungal strains used in antimicrobial assays, *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC2592), *Bacillus pyocyaneus* (CMCCB10104), *Bacillus megatherium*, *Bacillus dysenteriae*, *Candida albicans* (ATCC2002), *Aspergillus flavus* (IFFI4015), and *Penicillium uticale* (IFFI2001), were obtained from Kunming Medical College. Bacteria were first grown in LB (Luria-Bertani) broth to an $OD_{600\,nm}$ of 0.8. A 10 μL aliquot of the bacteria was then taken and added to 8 mL of fresh LB broth with 0.7% agar and poured over on a 90 mm Petri dish containing 25 mL of 1.5% agar in LB broth. After the top agar hardened, a 20 μL aliquot of the sample filtered on a 0.22 μm Millipore filter was

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dropped onto the surface of the top agar and completely dried before incubated overnight at 37℃. If an examined sample contains antimicrobial activity, a clear zone formed on the surface of the top agar representing inhibition of bacterial growth. Minimal inhibitory concentration (MIC) was determined in liquid LB medium by incubating the bacteria in LB broth with variable amounts of the sample tested. The MIC at which no visible growth occurred was recorded. In the assays of anti-fungi activity, for strains of *A. flavus* and *P. uticale*, the fungal spore concentration was first counted under a microscope. The fungi with an initial concentration of 10^5 spores/mL were cultured with variable amounts of the sample tested in yeast extract-peptone-dextrose broth.

1.9 Cytotoxicity on human cancer cell lines

Hematopoietic cell line: K562 (human blastic crisis-chronic myelogenous leukemia), and large intestine tumor cell line, HCT-15, were obtained from Chinese Type Culture Collection (Kunming Institute of Zoology, the Chinese Academy of Sciences). Cells were cultured in PRMI-1 640 media (Gibco, Grand Island NY) at 37℃, 5% CO_2, supplemented with 15% heat-inactivated new-born calf serum and for time intervals of 48-72 h. The samples were filtered on 0.22 μm Millipore filters and diluted to desired concentrations with the culture media. Trypsinized cells were dispensed into each well of a Costar flat-bottom culture plate (Costar, Charlotte, NC). After a 24 h culturing, the samples dissolved in physiological saline solution (PSS) were added with various concentrations. Following incubation, cell growth was measured by microculture tetrazolium assay method (MTT, Mosmann, 1983). PSS was used as the control.

2 Results and Discussion

2.1 Acute toxicity

Mice injected with the skin secretions of *T. verrucosus*, *B. maxima* and *B. andrewsi* exhibited typical poisoned symptoms, especially mice injected with the skin secretions of *T. verrucosus* and *B. maxima*. The symptoms of gasp, jerk, ventral decubitus and lacrimation, were observed in the mice injected with *T. verrucosus* skin secretions, and the symptoms of gasp, tension, jerk, ataxia and lacrimation were observed in the mice injected with *B. maxima* skin secretions. The LD_{50} (intraperitoneal) values of the skin secretions of *T. verrucosus*, *B. maxima*, and *B. andrewsi* were 11.5 mg/kg, 18.8 mg/kg, 264 mg/kg, respectively (Table 1). Injection of the skin secretions of *R. nigromaculata*, *R. guentheri* and *R. limnocharis* resulted in neither obvious poisoned symptoms normortality in mice for the doses up to 500 mg/kg, indicating that they are nontoxic. The LD_{50} values of the skin secretions of *T. verrucosus* and *B. maxima* on mice were in the same grade as those of Viperidae snake venoms (1-30 mg/kg) (Broad *et al.*, 1979). After incubated at 100℃ for 10 min, they lost their ability to cause mortality in mice, indicating that the lethal components in the secretions are heat unstable. Furthermore, after gel filtration of the skin secretions of *T. verrucosus* and *B. maxima* on a Sephadex G-75 (Superfine, Pharmacia) column, it was revealed that the lethal activities of these secretions were caused by protein and peptide fractions with the molecular weights ranging from 3 to 60 kDa (data not shown). This is similar to the situation in snake venoms. Most toxic components of snake venoms are protein and peptides, and many of them are heat unstable. In a common view, toxic substances from amphibians are small molecular weight components, such as biogenic amines, alkaloids, steroids, steroid esters, tetrodotoxin and small peptides (Trevor, 1985; Daly, 1978; Daly, 1995). This is the first report that components with high molecular weights from amphibians are highly toxic to mammals. The toxic components of these skin secretions, their biochemical structures and mechanism of actions are currently under investigation. On the other hand, the lethal toxicity of the skin secretions of *B. andrewsi* was not affected after incubated at 100℃ for 10 min. Comparatively, the lethal activities of the skin secretions of *T. verrucosus* and *B. maxima* were 20-30 times higher than that of the skin secretions of *B. andrewsi*. Different from the situation in *T. verrucosus* and *B. maxima* skin secreations, after

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Table 1 Acute toxicity of amphibian skin secretions and comparison with those of some snake venoms (intraperitoneal)

Animal species	LD_{50} (mg/kg)	Data source
Amphibia		
Salamandridae		
T. verrucosus	11.5 ± 2.1	This report
Discoglossidae		
B. maxima	18.75 ± 4.4	This report
Bufonidae		
B. Andrewsi	264 ± 53	This report
Ranidae		
R. nigromaculata	—	This report
R. guentheri	—	This report
R. limnocharis	—	This report
Reptile		
Elapidae		
Naja naja	0.565	Broad *et al.* (1979)
Ophiophagus hannah	1.8	Broad *et al.* (1979)
Viperidae		
Crotalus adamanteus	11.4	Broad *et al.* (1979)
Demansia olivacea	14.2	Broad *et al.* (1979)
Bothrops atrox	27.8	Broad *et al.* (1979)

The LD_{50} values of three determinations, including standard deviations, were shown in the Table. — means no detectable mortality in the doses up to 500 mg/kg.

gel filtration of the skin secretions of *B. andrewsi* on a Sephadex G－25 (Fine, Pharmacia) column, it was shown that the lethal activity of these secretions was caused by small molecular weight fractions (< 1 kDa), similar to well-known bufotoxins (data not shown).

2.2 Enzymatic activity assay

All of the six skin secretions tested displayed proteolytic activity on casein, as shown in Fig. 1. Different from snake venoms, in which phospholipase A_2 activity is a common activity, and except the skin secretions of *T. verrucosus*, other five skin secretions did not exhibit phospholipase A_2 activity in our assay conditions. In the same time, acetylcholine esterase activity was not found in all the six skin secretions assayed.

2.3 Protease inhibitory activity

The protease inhibitory activity was determined by inhibitory effects of the secretions on the amidolytic activities of trypsin and thrombin. Interestingly, all the six skin secretions exhibited trypsin inhibitory activity, providing the evidence that there are potent trypsin inhibitors contained in the secretions. The skin secretions at a concentration of 200 μg/mL inhibited 90% － 100% of the amidolytic activity of trypsin (0.5 μg/mL

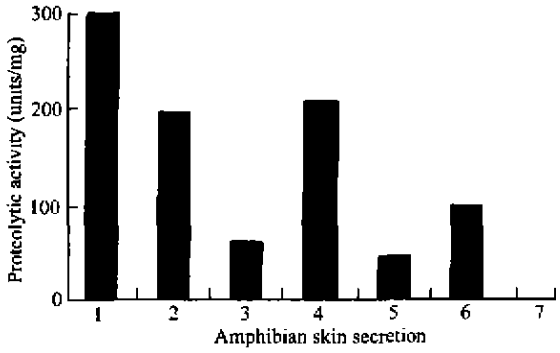

Fig. 1 Proteolytic activity of amphibian skin secretions
Proteolytic activity was assayed according to the method of Saleemaddin *et al.* (1980). One unit of the enzyme is the protein amount that causes a decrease of 0.01 absorbance unit in 10 min at 595 nm. 1. *B. maxima*; 2. *B. andrewsi*; 3. *T. verrucosus*; 4. *R. guentheri*; 5. *R. nigromaculata*; 6. *R. limnocharis*; 7. Control.

final concentration). In contrasts, for thrombin, these skin secretions showed no distinctive inhibition activity.

Of interest is the fact that all these six amphibian skin secretions exhibited trypsin inhibitor activity. Up to now, only a serine protease inhibitor (named as BSTI, Mignogna *et al.*, 1996) was purified and characterized in amphibian skin secretions. Our current study demonstrated that all these six amphibian skin secretions possessed trypsin inhibitory activity, which made it difficult to say the phenomenon was only a coincidence. In fact, we had identified a novel protein serine protease inhibitor with a molecular weight of 67 kDa from *B. maxima* skin secretions, and several other peptidic trypsin inhibitors in the same skin secretions (Lai *et al.*, 2002).

2.4 Effects on blood coagulation system

All of the six amphibian skin secretions did not display significant effects on blood coagulation system. Incubated with citrated human platelet-poor plasma, in the presence or absence of $CaCl_2$, they showed neither procoagulant nor anticoagulant activities to citrated human platelet-poor plasma and platelet-rich plasma. Further, no coagulating activity was found in these skin secretions.

2.5 Hemolysis and hemorrhagic activity

All of the six skin secretions did not possess hemorrhagic activity on mice even in a dose up to 10 μg/g

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Table 2 Antimicrobial activity of amphibian skin secretions

Microbes	Minimal inhibitory concentration (μg/mL)	
	B. maxima	*T. verrucosus*
Bacteria		
E. coli (ATCC25922)	20	—
S. aureus (ATCC2592)	20	50
B. pyocyaneus (CMCCB10104)	40	100
B. megatherium	20	50
B. dysenteriae	20	50
Fungi		
C. albicans (ATCC2002)	20	50
A. flavus (IFFI4015)	80	—
P. uticale (IFFI2001)	—	—

— means no detectable antimicrobial activity in a dose up to 200 μg/mL. The skin secretions from *R. nigromaculata*, *R. limnocharis* and *R. guentheri* had no antimicrobial activity at 200 μg/mL. The data represent the mean of three determinations, which differed by 10% – 15%.

body weight. This result implies that, unlike in snake venoms, there might be no metalloproteinase hemorrhagin-like components in amphibian skin secretions. On the other hand, in a dose up to 20 μg/mL, only the skin secretions of *B. maxima* caused 25% hemolysis of rabbit blood red cells. The other five skin secretions possessed no hemolytic activity in the same dosage.

2.6 Antimicrobial activity

Several amphibian skin secretions have been analyzed in detail and found to contain a large number of different antimicrobial peptides, which represent the effector molecules of innate immunity. Furthermore, the sensitivities of different bacteria and fungal strains to antimicrobial peptides from various amphibian species are different (Clarke, 1997; Charpentier *et al.*, 1997; Mangoni *et al.*, 2000; Goraya *et al.*, 2000). In this line, as listed in Table 2, the skin secretions of *T. verrucosus*, *B. maxima* possessed significant antimicrobial activities on tested bacteria and fungal strains, But the sensitivities of the bacteria and fungal strains assayed to these skin secretions were different. On the other hand, the antimicrobial activity was not detected in the skin secretions of *B. andrewsi*, *R. nigromaculata*, *R. guentheri* and *R. limnocharis* in this assay system. There are several possibilities to explain this phenomenon. One is that, although there are antimicrobial components in these three skin secretions, the microbial strains used in these assays are not sensitive to them. The other one is that the antimicrobial components responsible for their innate immunity to microbes are not secreted out but kept in the skin to perform their biological functions. This is the situation in *B. andrewsi*. The homogenized skin samples of *B. andrewsi* possessed antimicrobial activity in the same assay system but the corresponding skin secretions did not. A third, the antimicrobial components were destroyed by proteases in the process of sample collection as described by Gibson *et al.* (1991).

2.7 Cytotoxicity on human cancer cell lines

As shown in Table 3, the skin secretions of *B. maxima*, *B. andrewsi* inhibited significantly the growth of both human hematopoetic and solid tumor cell lines. In a dose of 20 μg/mL, an average of 30% cell growth inhibition was observed for *B. andrewsi* skin secretions, and an average of 48% cell growth inhibition was observed for *B. maxima* skin secretions. This is in agreement with the fact that dried *B. andrewsi* toad skin secretions are widely used as an antitumor drug in traditional oriental medicine. It has been reported that magainins, the anti-microbial pep tides isolated from *Xenopus laevis* skin, possess antitumor activity (Cruciani *et al.*, 1991; Ohsaki *et al.*, 1992). It is very interesting to go further for answering

Table 3 *In vitro* cytotoxicity of amphibian skin secretions

Skin secretions	Cancer cell lines			
	K562		HCT – 15	
	Cell growth (A570)	Inhibition (%)	Cell growth (A570)	Inhibition (%)
Control	1.067 ± 0.054		1.134 ± 0.032	
B. maxima	0.562 ± 0.083	47.3	0.593 ± 0.057	47.7
B. andrewsi	0.740 ± 0.097	30.7	0.732 ± 0.033	34.5

Cell growth was measured by microculture tetrazolium assay method (MTT, Mosmann, 1983). The amphibian skin secretion concentration is 20 μg/mL. Cancer cells are 4×10^5 cells/mL. The data represent the mean ± SD of four determinations.

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the question if the cytotoxicity of skin secretions on human cancer lines is contributed by their anti-microbial components or by other specific constituents.

References:

Berman J D. 1973. Structural properties of acetylcholinesterase from eel electric tissue and bovine erythrocyte membranes[J]. *Biochemistry*, **12**:1710 – 1714.

Bevins C L, Zasloff M. 1990. Peptides from frog skin[J]. *Annu. Rev. Biochem.*, **59**:395 – 414.

Bignami G S. 1993. A rapid and sensitive hemolysis neutralization assay for palytoxin[J]. *Toxicon*, **31**:817 – 820.

Broad A J, Sutherland S K, Coulter A R. 1979. The lethality in mice of dangerous Australian and other snake venoms[J]. *Toxicon*, **17**:661 – 664.

Charpentier S, Amiche M, Mester J, *et al*. 1997. Structure, synthesis, and molecular cloning of dermaseptins B, a family of skin peptide antibiotics[J]. *J. Biol. Chem.*, **273**:14690 – 14697.

Clarke B T. 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications[J]. *Biol. Rew.*, **72**:365 – 379.

Cruciani R A, Barker J L, Zasloff M, *et al*. 1991. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation[J]. *Proc. Natl. Acad. Sci. USA*, **88**:3792 – 3796.

Daly J W, Brown G B, Mensah-Dwumah M, *et al*. 1978. Classification of skin alkaloids from neotropical poison-dart frogs (Dendrobatidae)[J]. *Toxicon*, **16**:163 – 188.

Daly J W, Myers C W, Whittaker N. 1987. Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substance in the amphibia[J]. *Toxicon*, **25**:1023 – 1095.

Daly J W. 1995. The chemistry of poisons in amphibian skin[J]. *Proc. Natl. Acad. Sci. USA*, **92**:9 – 13.

Erspamer V, Melchiorri P, Erspamer G F, *et al*. 1985. Phyllomedusa skin: a huge factory and store-house of variety of active peptides[J]. *Peptides*, **6**:7 – 12.

Gibson B W, Tang D, Mandrell R, *et al*. 1991. Bombinin-like peptides with antimicrobial activity from skin secretions of the Asian toad, *Bombina orientalis*[J]. *J. Biol. Chem.*, **266**:23103 – 23111

Goraya J, Wang Y Q, Li Z H, *et al*. 2000. Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs *Rana luteiventris*, *Rana berlandieri* and *Rana pipiens*[J]. *Eur. J. Biochem.*, **267**:894 – 900.

Haas G H de, Postema N M, Nieuwenhuizen W, *et al*. 1968. Purification and properties of a phospholipase A from porcine pancreas[J]. *Biochim. Biophys. Acta*, **159**:103 – 117.

Lai R, Liu H, Lee W H, *et al*. 2002. Identification and cloning of a trypsin inhibitor from skin secretions of Chinese red-belly toad *Bombina maxima*[J]. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, **131**:47 – 53.

Lazarus L H, Attila M. 1993. The toad, ugly and venomous, wears yet a precious jewel in his skin[J]. *Prog. Neurobiol.*, **41**:473 – 492.

Macleod R J, Lembessis P, James S, *et al*. 1998. Isolation of a member of the neurotoxin/cytotoxin peptide family from *Xenopus laevis* skin which activates dihydropyridine sensitive Ca^{2+} channels in mammalian epithelial cells[J]. *J. Biol. Chem.*, **273**:20046 – 20051.

Mangoni M L, Rinaldi A C, Giulio A D, *et al*. 2000. Structure-function relationships of temporins, small antibacterial peptides from amphibian skin[J]. *Eur. J. Biochem.*, **267**:1447 – 1454.

Meier J, Theakston R D. 1986. A proximate LD_{50} determinations of snake venoms using eight to ten experimental animals[J]. *Toxicon*, **24**:395 – 401.

Mignogna G, Pascarella S, Wechselberger C, *et al*. 1996. BSTI, a trypsin inhibitor from skin secretions of *Bombina bombina* related to protease inhibitors of nematodes[J]. *Protein Sci.*, **5**:357 – 362.

Mollay C, Wechselberger C, Mignogna G, *et al*. 1999. Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats[J]. *Eur. J. Pharmacol.*, **374**:189 – 196.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays[J]. *J. Immunol. Methods*, **65**:55 – 63.

Ohsaki Y, Gazdar A F, Chen H C, *et al*. 1992. Antitumor activity of magainin analogues against human lung cancer cell lines[J]. *Cancer Res.*, **52**:3534 – 3538.

Ownby C L, Bjarnason J, Tu A T. 1978. Hemorrhagic toxins from rattlesnake (*Crotalus atrox*) venom: Pathogenesis of hemorrhage induced by three purified toxins[J]. *Am. J. Pathol.*, **93**:201 – 218.

Saleemuddin M, Ahmad H, Husain A. 1980. A simple, rapid, and sensitive procedure for the assay of endoprotease using Commassie brilliant blue G – 250[J]. *Anal. Biochem.*, **105**:202 – 206.

Seon A A, Pierre T N, Redeker V, *et al*. 2000. Isolation, structure, synthesis, and activity of a new member of the calcitonin gene-related peptide family from frog skin and molecular cloning of its precursor[J]. *J. Biol. Chem.*, **275**:5934 – 5940.

Trevor J C. 1985. Poisonous frogs[A]. In: Frogs & Toads[M]. London: Whittet Books Ltd. 58 – 68.

Zhang Y, Wiser A, Xiong Y L, *et al*. 1995. A novel plasminogen activator from snake venom[J]. *J. Biol. Chem.*, **270**:10246 – 10255.